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Combined hydration and antibiotics with lisinopril to mitigate acute and delayed high-dose radiation injuries to multiple organs

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Abstract

The NIAID Radiation and Nuclear Countermeasures Program is developing medical agents to mitigate the acute and delayed effects of radiation that may occur from a radionuclear attack or accident. To date, most such medical countermeasures have been developed for single organ injuries. Angiotensin converting enzyme (ACE) inhibitors have been used to mitigate radiation-induced lung, skin, brain and renal injuries in rats. ACE inhibitors have also been reported to decrease normal tissue complication in radiation oncology patients. In the current study we have developed a rat partial-body irradiation (leg-out PBI) model with minimal bone marrow sparing (one leg shielded) that results in acute and late injuries to multiple organs. In this model, the ACE inhibitor lisinopril (at $\sim 24 \text{ mg m}^{-2} \text{ day}^{-1}$ started orally in the drinking water at 7 days after irradiation and continued to 150 days) mitigated late effects in the lungs and kidneys after 12.5 Gy leg-out PBI. Also in this model, a short course of saline hydration and antibiotics mitigated acute radiation syndrome following doses as high as 13 Gy. Combining this supportive care with the lisinopril regimen mitigated overall morbidity for up to 150 days after 13 Gy leg-out PBI. Furthermore lisinopril was an effective mitigator in the presence of the growth factor G-CSF ($100 \mu\text{g kg}^{-1} \text{ day}^{-1}$ from days 1-14) which is FDA-approved for use in a radionuclear event. In summary, by combining lisinopril (FDA-approved for other indications) with hydration and antibiotics, we mitigated acute and delayed radiation injuries in multiple organs.

Keywords

Lisinopril; pneumonitis; nephropathy; supportive care; granulocyte-colony stimulating factor (G-CSF); Enrofloxacin

Introduction

The aim of the NIAID Radiation and Nuclear Countermeasures Program is to develop medical countermeasures to treat the acute and delayed effects of radiation that may occur from a nuclear attack or terrorist event (DiCarlo et al. 2008, 2011). This includes acute radiation syndrome (ARS) for injury to the gastrointestinal (GI) and hematopoietic systems within 30 days of exposure and delayed effects of acute radiation exposure (DEARE) for injury to the lungs, kidneys and brain as well as damage to the gut and hematopoietic systems that may manifest months after exposure. In a nuclear or radiological event, irradiation injuries to multiple organs will need to be addressed.

In the past 10 years a number of investigators, including our group have developed animal models of radiation injury to single organs in order to test medical radiation countermeasures for the NIAID program. Since the bone marrow is one of the most sensitive early targets of radiation, many animal models have been developed to study this aspect of ARS. The most popular is a mouse model of total body irradiation (TBI) (Plett et al. 2012, 2015; Chua et al. 2012; Williams et al. 2010).

In order to develop models of DEARE, bone marrow toxicity must be avoided in one of three ways: (i) mitigation by drugs, (ii) bone marrow transplantation or (iii) sparing the marrow by shielding. We developed a rat model of TBI with 11.5 Gy and followed by a bone marrow transplant (Medhora et al. 2014; Moulder et al. 2014). However, bone marrow transplants will not be feasible in the case of mass casualty attacks. Sparing of some bone marrow can be expected in a nuclear event since partial body exposures are likely especially after a nuclear accident. Accordingly we have developed a relevant partial body irradiation (PBI) model shielding one hind leg (which will be described as 'leg-out PBI'). This is similar to the PBI/BM5 models developed for mouse (Booth et al. 2012) and non-human primates (NHP) (MacVittie et al. 2012). The mouse and NHP models have been used to investigate radiation injury to single organs such as the GI system (Booth et al. 2012) or the lung (MacVittie et al. 2012).

Using such animal models, investigators have proceeded to test countermeasures to mitigate radiation damage. Progress has been made to mitigate radiation-induced bone marrow toxicity using hematopoietic growth factors especially granulocyte-colony stimulating factor (G-CSF) (MacVittie et al. 2005). G-CSF and its pegylated versions are the only mitigators that have been approved by the FDA to reduce ARS when started at least 24 hours after a single dose of radiation (FDA 2015). This drug has been included in the Strategic National Stockpile. Approval for G-CSF for ionizing radiation-induced injury was pursued under the 'FDA Animal Rule' (Crawford. 2002) since it is not ethical to conduct a clinical trial after single doses of radiation that will induce bone marrow failure (Farese and MacVittie. 2015). The use of G-CSF is often accompanied by 'supportive care' in the first few weeks after PBI. Supportive care consists of parenteral fluids and antibiotics to address acute GI and bone marrow toxicity after radiation and can be provided with allocation of minimal resources. The LD₅₀ (radiation dose resulting in 50% death) increased from 4.1 Gy at Nagasaki (n=75, (Levin et al. 1992)) to 8.9 Gy at Chernobyl (n=238, (Anno et al. 2003)) which was largely ascribed to the use of intensive supportive care. In non-human primates

(Chinese Macaques) population-based supportive care improved the LD₅₀ from 4.9 Gy to 6.6 Gy (Yu et al. 2015), while symptom-based supportive care that included blood transfusion increased the LD₅₀ to 7.4 Gy (Yu et al. 2015). In primate studies and in the clinic, steroids have also been used to manage symptoms of radiation pneumonitis (Yu et al. 2015, Garofola et al. 2014 a & b, Geraci et al. 1992, Gross 1980, Gross et al. 1988). Although few studies have addressed the effect of supportive care on DEARE, indirect evidence from accidents (e.g. Tokai-Mura) and the bone-marrow transplant patient population show little mitigation of late effects, especially with respect to lung and kidney injury. It would be surprising if supportive care did not show a beneficial effect on radiation-induced late effects; but the exact role it plays and the best approach to follow needs to be addressed as the community moves forward in assessing medical countermeasures (MCM) efficacy in appropriate animal models.

We have previously used a 11.5 Gy TBI plus bone marrow transplantation model to study mitigation of injury to the lung and kidney in the same rat by three angiotensin-converting enzyme (ACE) inhibitors (enalapril, captopril and fosinopril) (Medhora et al. 2014) and other countermeasures (Gao et al. 2012). In addition, incidental use of ACE inhibitors, particularly lisinopril reduced the occurrence of radiation pneumonitis in cancer patients (Jenkins and Watts. 2011; Jenkins and Welsh. 2011; Kharofa et al. 2012). Others have examined ACE inhibitors for radioprotection as well as mitigation of ARS, including hematopoietic injury (Day et al. 2013; Davis et al. 2010). To advance our studies for the NIAID program, we are using the ACE inhibitor lisinopril in a rat leg-out PBI model with doses as high as 13 Gy to examine non-hematopoietic damage. In addition to lisinopril, the combined use of saline and antibiotic is being tested as a variable, to determine if they effect mitigation by lisinopril.

Materials & Methods

Animal care

All animal protocols were approved by Institutional Animal Care and Use Committees (IACUC) at the Medical College of Wisconsin. Based upon direction from the IACUC, rats were designated as morbid and euthanized and followed by necropsy if they met specified veterinarian's criteria as described previously (Medhora et al. 2015).

Animals and Irradiation

Leg-out PBI in rats—WAG/RijCmcr female rats were irradiated without the use of anesthetics at 11-12 weeks of age, they had a weight of ~155 grams. All irradiations were done between 9 – 11 am. For leg-out PBI, non-anesthetized rats were immobilized in a plastic jig and irradiated using a XRAD 320KV orthovoltage x-ray system. The x-ray system was operated at 320 kVp and 13 mAs with a half value layer of 1.4 mm Cu with a dose-rate of 1.75 Gy min⁻¹ for total doses from 11-13.5 Gy. During the irradiation, each rat was confined in a chamber which allows irradiation of 2 rats simultaneously. One hind limb of each rat was carefully externalized from the chamber and shielded with a 0.25 inch lead block. The dose to this leg was 2 Gy. The dual-chambered jig was placed on a plane perpendicular to the beam direction, with distance from source to the midline of rats set at

61 cm. Collimator jaws and dosimetry were used as previously described (Medhora et al. 2014). The irradiation field at midline was large enough to cover both chambers with adequate (at least 2 cm) margins.

Mitigation of late injuries to the lungs and kidneys after 12.5 Gy leg-out PBI

30 rats underwent 12.5 Gy leg-out PBI. They were randomized to the following groups (1) no intervention (n=10), (2) lisinopril ($\sim 24 \text{ mg m}^{-2} \text{ day}^{-1}$) starting from day 7 after irradiation and continuing until study termination (n=10). Lisinopril was delivered in drinking water at a concentration of 40 mg lisinopril/liter (Kma et al. 2012, Moulder et al. 2014). This dose is comparable to that approved for clinical use on a $\text{mg m}^{-2} \text{ day}^{-1}$ basis. (3) Lisinopril (in the same dose and schedule as group (2)) along with G-CSF (Filgrastim; Amgen, Inc, Thousand Oaks, CA) administered subcutaneously at a dose of $100 \mu\text{g kg}^{-1}$ once a day from days 1-14 after radiation and Enrofloxacin ($10 \text{ mg kg}^{-1} \text{ day}^{-1}$) from days 2-28 in the drinking water (n=10). Two additional groups (4&5) included age matched control rats with no irradiation and no intervention (0 Gy, n=6) and no irradiation with lisinopril in the same dose and schedule as group (2) (n=6). The rats in Groups 4 and 5 (controls) did not become morbid and were not shown in the Kaplan-Meier graphs. All rats were followed for at least 150-day survival.

Mitigation of ARS+DEARE after 13.0 Gy leg-out PBI with fluids, antibiotic and lisinopril

40 rats were given 13.0 Gy leg-out PBI plus supportive care. Supportive care consisted of Enrofloxacin $10 \text{ mg kg}^{-1} \text{ day}^{-1}$ from days 2-28 and hydration by daily subcutaneous injection of saline $40 \text{ ml kg}^{-1} \text{ day}^{-1}$ from days 3-7. These were randomized into the following groups: (1) no additional treatments (n=14). (2) Lisinopril ($\sim 24 \text{ mg m}^{-2} \text{ day}^{-1}$) starting from day 7 after irradiation and continuing until study termination (n=13). (3) Lisinopril in the same dose and schedule as group (2) with G-CSF administered subcutaneously at a dose of $100 \mu\text{g kg}^{-1}$ once a day starting 24 hours after radiation and continuing for 14 days (n=13), to test if lisinopril mitigates DEARE when used with G-CSF. Additional non-irradiated groups are: (4) age matched control rats (0 Gy) with no intervention (n=3) and (5) no irradiation with supportive care (n=3). All rats were followed for 150-day survival.

Breathing interval measurements in rats

Breathing rates and body weights for each rat were measured every two weeks from 28-98 days after exposure as described previously (Medhora et al. 2012, 2014). Rats were restrained in a Plexiglas jig, which was placed in a transparent, airtight box. A differential pressure transducer was connected to a data acquisition device (Dataq-DI 158U, Dataq Instruments Inc., Akron, OH, USA) to sense changes in pressure in the box. The frequency of pressure changes was recorded and analyzed. Recordings for a maximum of 10 minutes per animal were used. The mean breathing rate for each rat was then calculated from four steady regions of the recording lasting greater than 15 seconds each. The inverse of the breathing rates were calculated to derive the breathing interval or time/breath in seconds. Since higher breathing rate and lower breathing intervals are associated with more lung damage, the breathing interval was set to 0 for all animals that died during pneumonitis to account for attrition (Medhora et al. 2012).

Lung histology

A subset of irradiated rats that were morbid by the euthanasia criteria between 42-70 days (n=6 rats) after 13 Gy leg-out PBI were randomly selected for histological evaluation of the lungs. The left lung was harvested, fixed and embedded in paraffin. Whole mount lung sections (4 μ m thick) were stained with hematoxylin and eosin and five randomly selected fields from different areas were scored in each lung. Foamy macrophages and alveolar wall thickness were scored as described (Medhora et al. 2014, 2015). Injury to the vasculature was measured on a 3 point scale as follows: No abnormal vessels (score 0), presence of some vessels with thickened walls but open lumens (see Fig. 6B) (score 1) and presence of vessel wall thickening with occluded lumens (score 2). Scores from irradiated morbid rats were compared to those from non-irradiated animals of the same strain (controls). Since no non-irradiated rats were morbid in this study, lungs from rats sacrificed at corresponding times in other experiments were used for comparison (controls). Scoring for all rats (morbid or non-irradiated controls) was done at the same time by one operator (JN) who was masked to the treatment groups.

Measurement of blood urea nitrogen (BUN)

Previous published work has shown that rising BUN levels are superior to histopathology for assessing radiation nephropathy (Moulder et al. 2011a). To measure BUN, rats were anesthetized with 3-5% isoflurane for blood draws by retro-orbital bleeds conducted by an experienced technician (Sharma et al. 2014). The BUN was assayed from serum as described previously (Medhora et al. 2014; Cohen et al. 1994) using a urease-nitroprusside colorimetric assay. BUN values were expressed as mg/dl of serum and median with 20-80% ranges were used for statistical analysis. Irradiated rats with BUN > 120 mg dl⁻¹ were euthanized and given a value of 120 mg dl⁻¹ to account for attrition, since such rats were previously confirmed to have severe and irreversible renal damage (Moulder et al. 1993).

Statistical analyses

Dose-response curves were fit by probit analysis and used to determine 50% lethal doses (LD₅₀). The significance of dose-response trends was assessed by the Mantel extension test; this is a non-parametric test of monotonic trends that does not (unlike probit analysis) assume an exact shape for the dose response. Morbidity data are shown by Kaplan Meier plots and tested for differences between groups by Peto-Peto Wilcoxon tests. Breathing intervals and BUN values are shown as medians and 20-80% ranges. Statistical difference of breathing intervals at each time point was measured by Kruskal-Wallis ANOVA on Ranks with multiple comparisons versus the control group by the Dunnett's method. Statistical differences between multiple groups for BUN were calculated by the Mann Whitney U test. Both the breathing interval and BUN analysis accounted for attrition. The T-test was used for two-group comparisons for scores for vascular wall thickness, alveolar wall thickness and macrophage counts between morbid rats versus matched, non-irradiated controls.

Results

1. Injury to multiple organs with increasing doses of leg-out PBI

Rats were irradiated with a single dose ranging from 11-13 Gy PBI keeping one hind leg out of the field as described. No interventions were given. At the 11 Gy dose all animals survived past 120 days after which they rapidly became morbid (Fig. 1) with increasing BUN levels. At 11.5, 12 and 12.5 Gy leg-out PBI, ~25% of rats were morbid by 20 days. This was followed by a second phase of morbidity between 50-70 days when rats had typical signs of pneumonitis with higher breathing rates. Pleural effusions were observed in some morbid rats on necropsy. Rats surviving past 70 days developed radiation nephropathy after 120 days. With 13 Gy leg-out PBI, there was GI morbidity with diarrhea between days 4-10 and only ~30% of the rats were alive at 20 days. As with other doses, these survivors ultimately became morbid from radiation pneumonitis (45-70 days) or nephropathy (120-160 days).

For ARS (death or morbidity requiring sacrifice prior to 30 days versus all animals treated) the dose-response was highly significant ($p=0.005$ by the Mantel extension test) and the LD_{50} was 12.7 (95% CI 12.3-13.4) Gy. The morbidity by ARS occurred between 5 and 17 days. For pneumonitis (death or morbidity requiring sacrifice prior to 95 days versus all animals that survived to 30 days) the dose-response was also highly significant ($p=0.003$ by the Mantel extension test), and the LD_{50} was 12.5 (95% CI 12.1-13.1) Gy. The pneumonitis morbidity requiring sacrifice occurred between 62 and 94 days. For assessing renal morbidity (animals surviving beyond 95 days and sacrificed because of high BUN) survival times were picked as all animals eventually were morbid between 125 and 164 days. The dose response did not reach statistical significance (all $p > 0.055$ by the Mantel extension test) for any choice of survival time.

2. Mitigation of lung and renal injury with lisinopril after 12.5 Gy leg-out PBI

We repeated the 12.5 Gy leg-out PBI studies because they exhibited multiple phases of injury with sufficient numbers remaining for evaluation of DEARE. We tested 3 groups of irradiated rats as described in the methods (also see Fig. 2). Rats (9 out of 10) in group 1) were morbid by 150 days. All rats from groups 2) or 3) were alive at this time and had BUN values $<55 \text{ mg dl}^{-1}$. Measurement of the breathing intervals (an indicator of lung function, Fig. 3) in randomly selected rats showed decrease at 56 and 70 days in group 1 (12.5 Gy leg-out PBI alone) as compared to either group with lisinopril at the corresponding time (Dunnett's method). As seen in Fig. 2, morbidity meeting the euthanasia criteria was only observed in this group corresponding with the timing for lung injury. There was an exponential increase in BUNs at 90, 120 and 150 days after irradiation in group 1) (black bars), with lower values in irradiated rats given lisinopril with or without G-CSF and Enrofloxacin (Fig. 4, $p<0.05$).

3. Mitigation of ARS after 13 Gy leg-out PBI with fluids and antibiotic

To mitigate the ARS after 13 Gy leg-out PBI, we included saline by subcutaneous injection from days 3-7 ($40 \text{ ml kg}^{-1}\text{day}^{-1}$) and Enrofloxacin ($10 \text{ mg kg}^{-1}\text{day}^{-1}$) from days 2-28 (see Methods). This supportive care reduced morbidity at 30 days after radiation ($p<0.01$) (Fig.

5), but morbidity due to radiation pneumonitis and nephropathy was still observed in these rats at later times (see dashed line 13 Gy+saline+Enro in Fig. 5).

4. Lisinopril combined with fluids and antibiotic mitigates ARS as well as renal injury

We combined the supportive care consisting of fluids and antibiotic as described above with lisinopril started from 7 days after 13 Gy leg-out PBI and continued thereafter. The combined regimen mitigated morbidity beyond 150 days after radiation as compared to no mitigators or supportive care ($p = 0.03$, Fig. 5). Histological examination of lungs of animals that became morbid between 42-70 days ($n=6$) showed increased alveolar wall thickness ($p<0.05$), vascular wall thickness ($p<0.001$) and macrophage infiltration ($p<0.05$) as compared to matched non-irradiated lungs ($n=4$) that were harvested for other studies from age-matched rats (historical controls; Fig. 6). Morbidity from lung injury was 26% for 13 Gy alone and 19% for 13 Gy plus lisinopril. While promising, the effect is not statistically significant ($p>0.25$ by either Chi-square or Fischer exact analysis). The median BUN of irradiated rats given supportive care only was also higher at 90, 120 and 150 days than those given lisinopril in addition to the supportive care as shown in Table 1.

Discussion

The radiosensitivity of cells and organs varies by dose and time to manifestation of injury after exposure, suggesting organ-specific mechanisms of radiation injury. Using partial body irradiation with shielding one leg of rats (partial femur, tibia, fibula and foot) with doses from 11.5 -13 Gy, we observed a sequelae of injuries. Additionally, we were able to mitigate injury for 150 days at doses at or above 11 Gy, indicating that the dose of ~2 Gy to one hind leg supported repopulation of the bone marrow since the LD_{50/30} in this strain of rats is around 7 Gy TBI (Moulder 2011b). With 12.5 Gy leg-out PBI up to 30% of rats were morbid by 80 days due to radiation pneumonitis, while surviving rats developed radiation nephropathy by 160 days (Figs. 1&2). This was accompanied by a decrease in breathing intervals at 56 days, a change that returned to baseline in surviving rats. The BUN of irradiated rats was increased above baseline at 90 days and reached morbid levels of 120 mg dl⁻¹ after 110 days indicating a chronic renal pathology (Fig.4). With 13 Gy leg-out PBI, >60% of animals were morbid at 4-10 days at which time they had diarrhea, strongly suggesting acute and lethal GI toxicity. This acute phase as well as delayed injury to the lungs occurs in a dose-dependent manner (*i.e.*, the dose response trends are statistically significant). Our results also demonstrate that when bone marrow in one leg is spared, the kidneys become the dose-limiting organ with no survival from radiation-nephropathy at the lowest dose we tested (11.5 Gy). Since GI injury is associated with bacterial translocation from the gut, we included an antibiotic from 2-28 days to address infection. We also included hydration with saline from days 3-7 to replenish fluid loss after 13 Gy leg-out PBI. This simple regimen of supportive care was able to suppress morbidity in the first 30 days at 13 Gy (Figs. 1&5) and we have observed similar results after 13.5 Gy (results not shown). Since bone marrow toxicity occurs at doses much lower than 11 Gy, we believe the acute effects we observed with 13 Gy leg-out PBI are primarily due to GI injury not hematopoietic injury. The shielding of one hind limb was sufficient to mitigate bone marrow toxicity even though it received 2 Gy. This evidence also supports our contention that ARS

following 13 Gy leg-out PBI is primarily due to GI toxicity. As with humans (Anno et al. 2003) and non-human primates (Yu et al. 2015), this study shows that saline hydration and antibiotics improved survival in rats after leg-out PBI. However, the primate studies were done without bone marrow sparing and therefore involved much lower doses of irradiation (<10 Gy). A study with male Wistar rats irradiated with 12 or 14 Gy PBI with both hind legs shielded (Boittin et al. 2015), supports the role of antibiotics for enhancing survival. That group detected bacterial infection in blood cultured pre-mortem following 14 Gy, in which rats not given Enrofloxacin died by 10 days, probably due to bacterial translocation from GI injury. In summary, our data show mitigation of ARS by saline and antibiotics and that the benefit of lisinopril for late injury to lungs and kidneys is not adversely impacted by this treatment.

We did not test the effect of supportive care with hydration or antibiotics alone on delayed effects of acute radiation exposure (DEARE) to the lungs or kidneys and we did not measure intake of lisinopril in irradiated rats. We have measured plasma renin activity (PRA) and water intake in irradiated rats given lisinopril and/or other ACE inhibitors in previous studies (Kma et al. 2012, Moulder et al. 2014). PRA is an indirect measure of circulating levels of ACE inhibitors. Further studies will be needed for FDA approval for lisinopril since at this time we do not know the actual dose of lisinopril delivered to each rat and have not yet met with the FDA for guidance for our models.

Late effects of radiation have been mitigated by us and others using two other ACE inhibitors, captopril and enalapril, implying this is a class effect of ACE inhibition, since these drugs have different structures and side groups but similar action on ACE. The mechanism for mitigation of radiation pneumonitis by lisinopril is complicated by the fact that ACE activity is decreased after radiation (Ghosh et al. 2009a). Additionally, higher doses of ACE inhibitors are more effective mitigators of pneumonitis (Ghosh et al. 2009b), suggesting other pathways besides engagement of angiotensin receptors. A number of pathways have been investigated for mitigation of radiation nephropathy by ACE inhibitors with most evidence supporting their action by suppression of angiotensin II-mediated activation of the AT1 (angiotensin II) receptor.

We have shown that by combining lisinopril with saline and antibiotics we can mitigate both the acute (GI) as well as the delayed effects of radiation. Since all three countermeasures we used, lisinopril, Enrofloxacin and saline are approved for clinical use, they can be prescribed immediately by practicing physicians or emergency medical staff authorization if needed, after a radiological attack or accident. In addition, we show that lisinopril is also effective in the presence of G-CSF and Enrofloxacin. There was no morbidity up to 150 days if lisinopril was given alone or with G-CSF and Enrofloxacin after 12.5 Gy leg-out PBI. Lisinopril was tested with G-CSF because the latter is approved by the FDA for use in irradiated subjects for hematopoietic toxicity from radiation and so is likely to be used in a radiological event. Our studies show that the mitigating action of lisinopril is not affected by use of this growth factor but we did not evaluate the effect of G-CSF on mitigation of radiation injuries in our rat model.

Countermeasures to be stockpiled and used for radiation injuries will need to be approved under the FDA Animal Rule (Crawford. 2002). This rule requires testing in animal models ‘...expected to react with a response predictive for human.....’. A number of single organ injury models were recommended early for the NIAID program (Williams et al. 2010). However, injuries to multiple organs are anticipated in a mass-casualty radio-nuclear event. Very few animal models with irradiation injuries to more than one organ covering ARS and DEARE including radiation nephropathy have been described. Models with PBI/BM5 (sparing 5% of the bone marrow) have been reported in mice (Booth et al. 2012) and non-human primates (MacVittie et al. 2012). The tibia, and feet of NHP (MacVittie et al. 2012) and tibias, fibulae and feet of mice (Booth et al. 2012) were shielded and the animals were anesthetized before or during irradiation. In this study we shielded one hind leg that included partial femur, tibia, fibula and foot. We did not anesthetize the rats to better simulate an unanticipated nuclear event. The NHP studies induced acute GI-ARS by 12 and 12.5 Gy PBI/BM5 after which 90% of the animals were morbid, before lung injury could manifest. The PBI/BM5 studies in mice delivered doses as high as 15.5 Gy but the results were confounded by ‘swollen muzzle’ syndrome in the first 30 days. (Booth et al. 2012; MacVittie et al. 2012). Our 13 Gy leg-out PBI model is the first we are aware of to include morbidity for lethal GI (part of ARS) as well as to test mitigators for radiation injury to the GI tract, lungs and kidneys. We have also observed increased risk of cardiac injury with 10 Gy PBI+BMT (Baker et al. 2009; Lenarczyk et al. 2013) and damage to the heart from pneumonitis after 15 Gy to the thorax only (Medhora et al. 2015). We are currently evaluating cardiac injury after 13 Gy leg-out PBI in rats. There is an obvious advantage to using a single agent such as lisinopril for mitigation of radiation injury to multiple radiosensitive organs. Meeting with the FDA and further research is needed to optimize this use of lisinopril and to test in combination with other agents that mitigate injuries from radiation. Our studies must be extended to include hematopoietic injury in cases of total body irradiation, to large animal models and to even higher doses of radiation that can be expected after a radiological attack or accident. And finally, mechanistic pathways of action of lisinopril in different organs after radiation must be explored to understand whether there is a common mechanism or diverse modes of action for different organ syndromes.

Conclusions

Currently there are few preclinical models for evaluation of both acute and delayed effects of radiation in the same animals. Also, most radiation countermeasures developed in the NIAID program to date have been tested only for efficacy for radiation-induced injuries in a single organ only. To further the development of a radiation countermeasures program we describe:

1. a rat model of non-hematopoietic injury to multiple organs after a single dose of radiation that can be followed up to 150 days
2. mitigation in this model, using countermeasures that are in use for other indications, that increases survival from 0 to >80%;
3. efficacy of a common FDA-approved agent, lisinopril, to mitigate DEARE.

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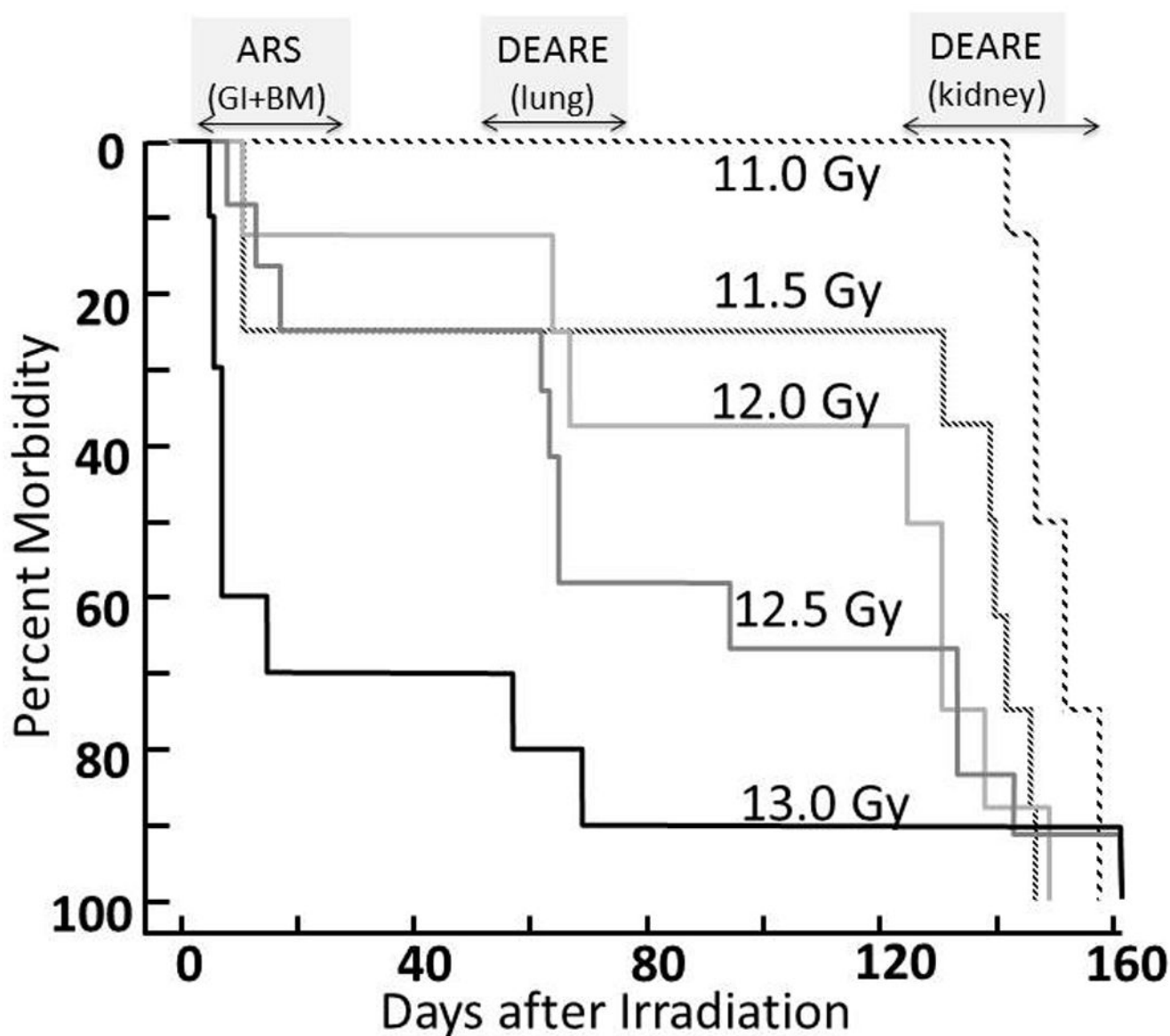


Fig. 1. Kaplan Meier plots for morbidity from increasing doses of partial body irradiation with shielding of one hind leg (leg-out PBI). The doses of leg-out PBI are indicated on each plot. The number of rats at risk (n) at 100 days for each dose is as follows: 11 Gy=8, 11.5 Gy=6, 12 Gy=5, 12.5 Gy=4, 13 Gy=1. Typical times for gastrointestinal (GI) and bone marrow (BM), lung or renal mortalities are indicated by arrows at the top.

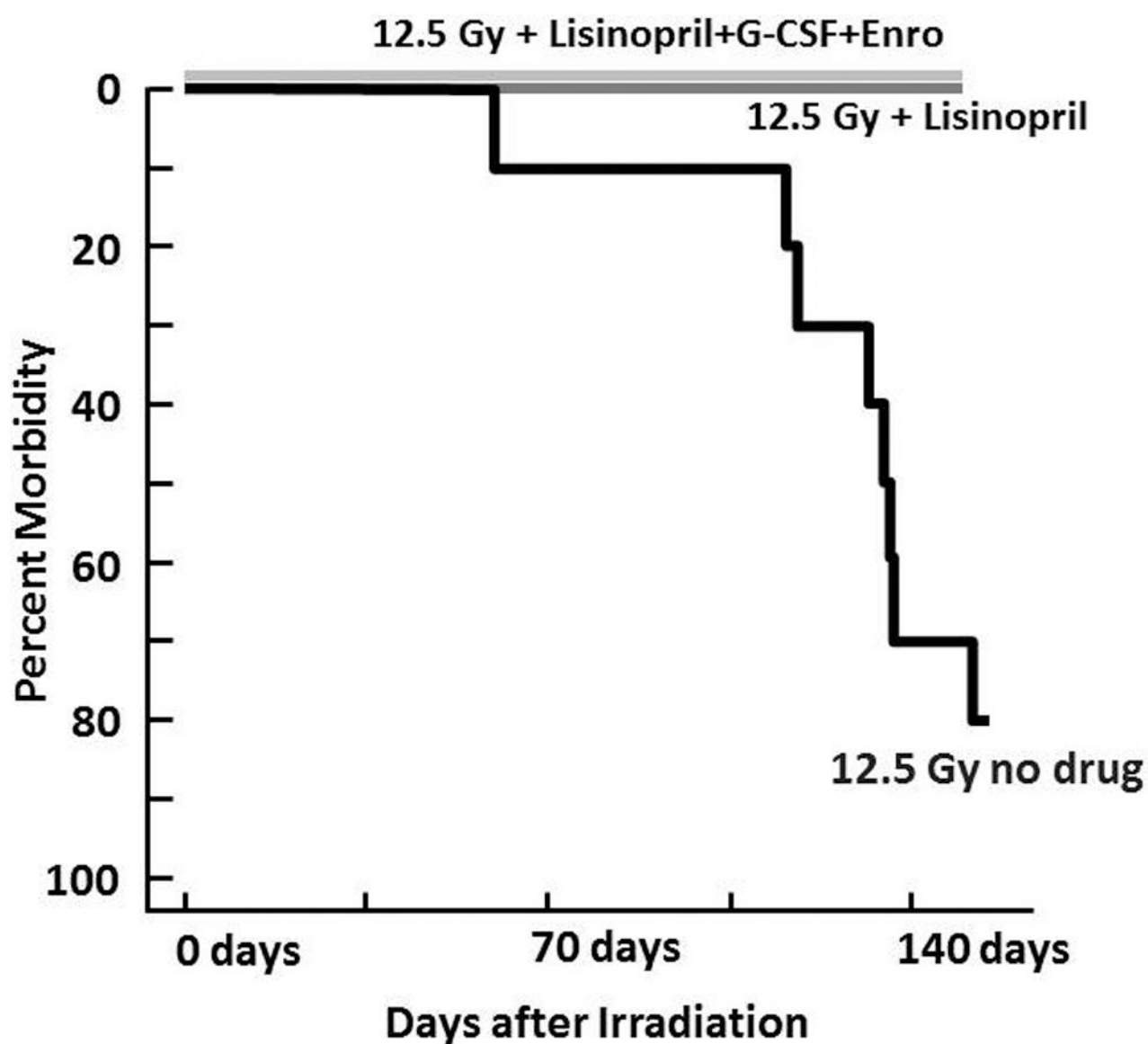


Fig. 2.

Mitigation of late effects after 12.5 Gy leg-out PBI. Kaplan Meier plots for morbidity after 12.5 Gy partial body irradiation with shielding of one hind leg (leg-out PBI) are shown. One group of rats was given lisinopril (at $24 \text{ mg m}^{-2} \text{ day}^{-1}$) started orally in the drinking water at 7 days after irradiation and continued to termination. A second group was given lisinopril with G-CSF and Enrofloxacin (Enro). As compared to 8 out of 10 irradiated rats with no drugs that were morbid by 150 days (black line), all rats given lisinopril alone ($p < 0.001$, $n=10$) or lisinopril with G-CSF and Enrofloxacin survived ($p < 0.001$, $n=10$) to 150 days.

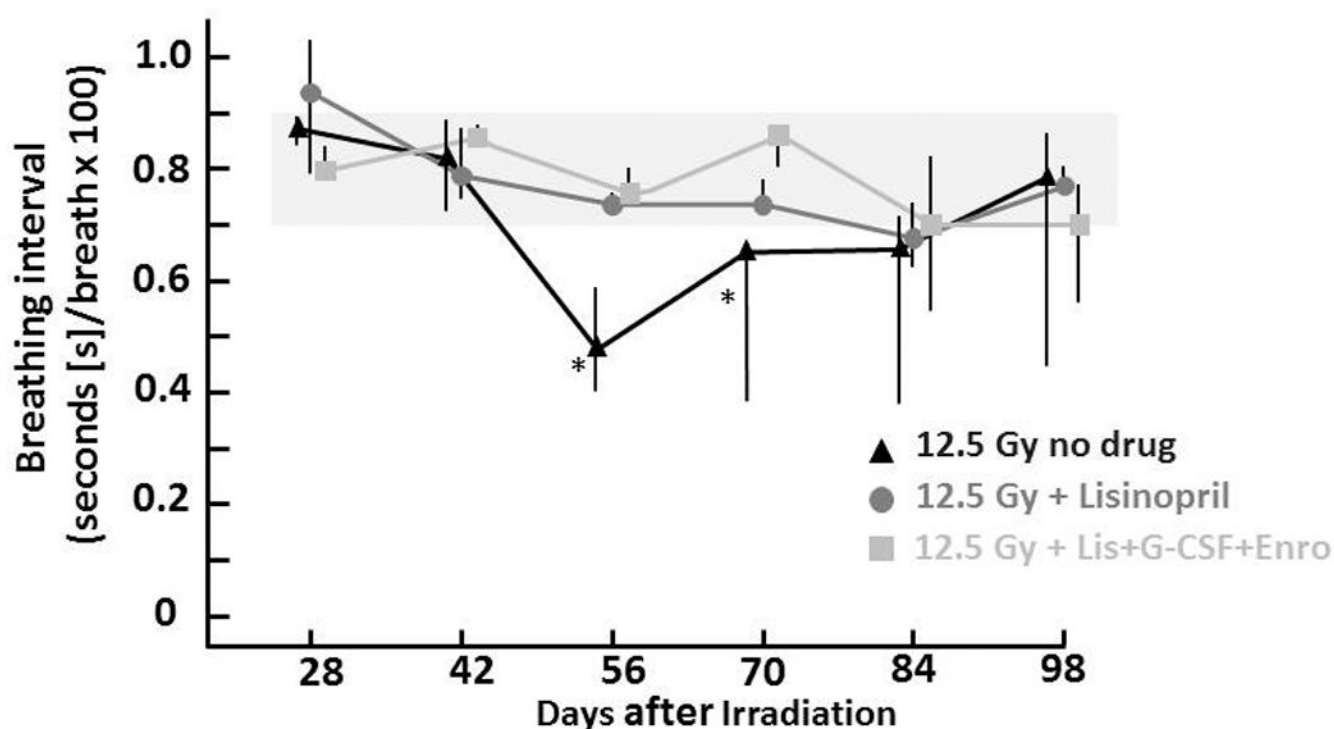


Fig. 3. Mitigation of radiation pneumonitis by lisinopril. The graph shows the changes in the breathing interval with time after 12.5 Gy partial body irradiation with shielding of one hind leg (leg-out PBI). The Y-axis shows medians and 20-80% ranges for time/breath in seconds X100. As in Fig. 2, one group of rats was given lisinopril ($\sim 24 \text{ mg m}^{-2} \text{ day}^{-1}$) started orally in the drinking water at 7 days after irradiation and continued to termination and another group was given lisinopril with G-CSF ($100 \mu\text{g kg}^{-1}$ from 1-14 days) and Enrofloxacin (grey line, Enro: $10 \text{ mg kg}^{-1} \text{ day}^{-1}$ from 2-28 days). The breathing interval was decreased ($p < 0.05$) at 56 days and 70 days in irradiated rats without drug when compared to irradiated rats given lisinopril ($n=4$ randomly selected rats per group). Morbid rats were assigned a breathing interval of 0 to account for attrition. The shaded bar in grey represents the range of breathing intervals X100 in non-irradiated controls.

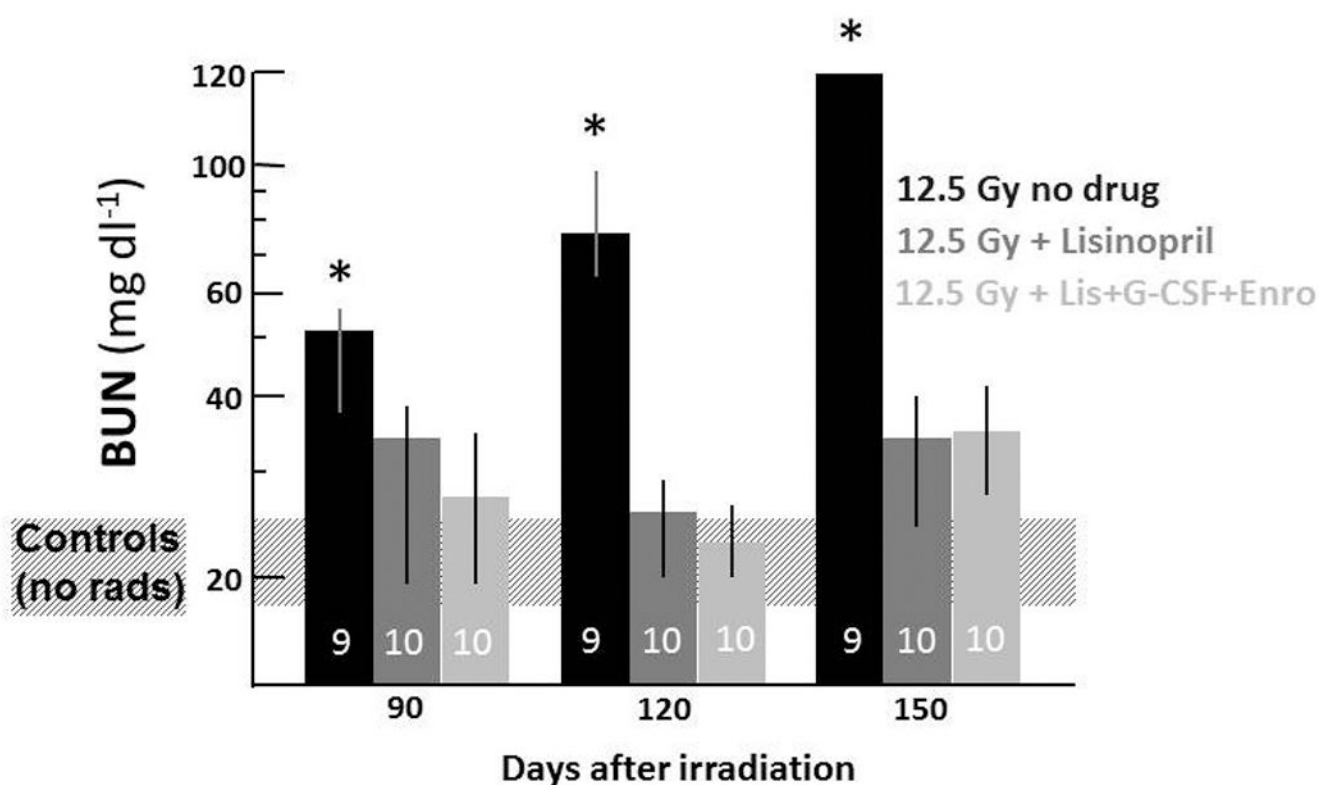


Fig. 4.

Mitigation of radiation nephropathy by lisinopril at $24 \text{ mg m}^{-2} \text{ day}^{-1}$ started orally in the drinking water at 7 days after irradiation and continued to termination. The graph shows the median and 20-80% ranges for blood urea nitrogen (BUN in mg dl^{-1}) in rats at 90, 120 and 150 days after 12.5 Gy partial body irradiation with shielding of one hind leg in rats (leg-out PBI). Black bars represent irradiated rats without intervention; grey bars represent rats given lisinopril and lisinopril with G-CSF and Enrofloxacin (Enro, light grey bars) as in Fig. 2 and 3. The Y-axis shows BUN values on a log scale. The numbers of rats in each group are included in each bar. Morbid rats with BUN 120 mg dl^{-1} were euthanized and assigned a BUN of 120 mg dl^{-1} to account for attrition. The horizontal hatched bar represents the range of BUNs in non-irradiated controls. * represent $p < 0.05$ as compared to corresponding groups with lisinopril. Note mitigation of the increase in BUN by lisinopril alone or in the presence of G-CSF and antibiotics at all 3 time points.

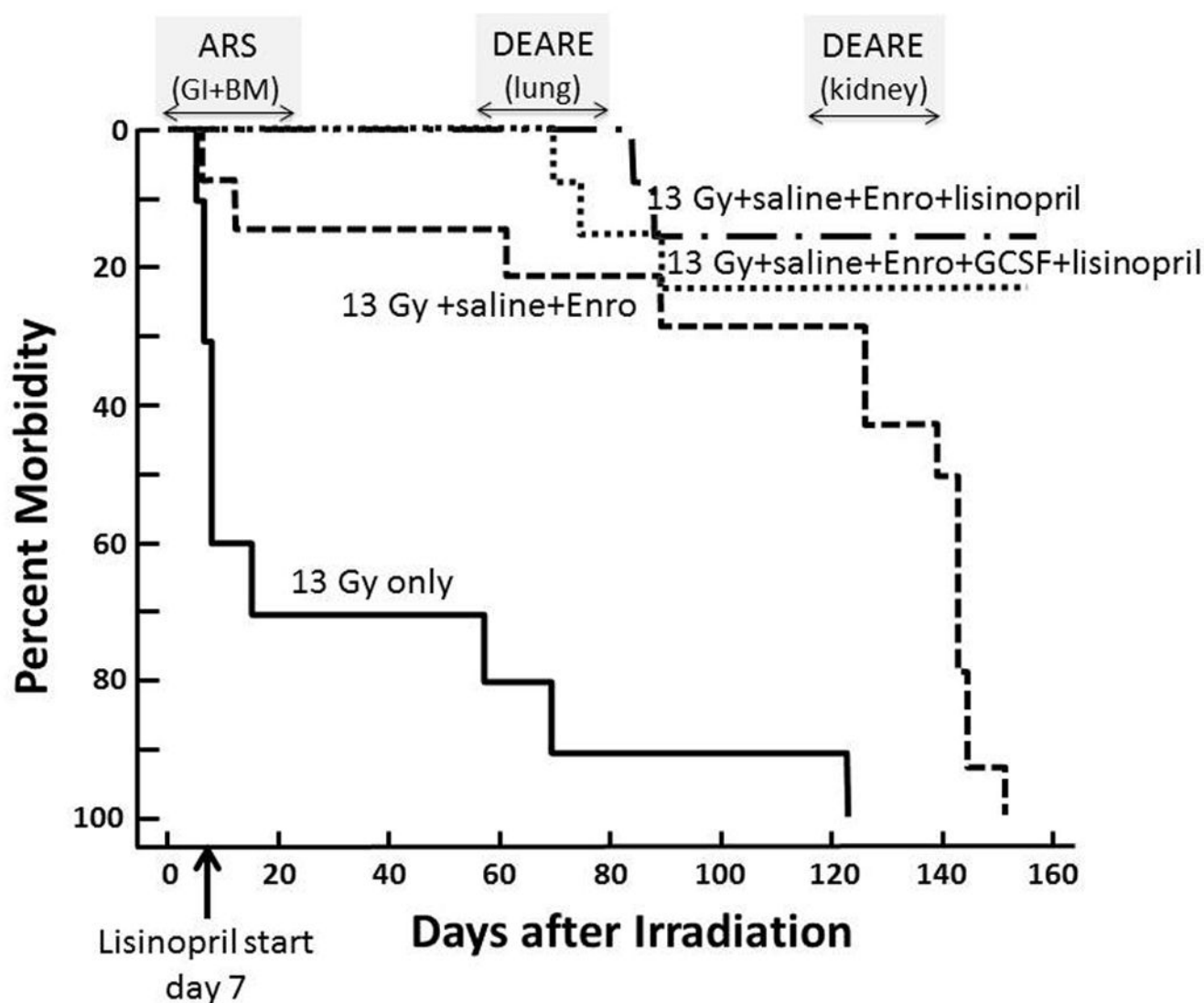


Fig. 5.

Mitigation of acute (ARS) and delayed (DEARE) radiation injuries by subcutaneous saline, antibiotics and lisinopril. Kaplan Meier plots show morbidity after 13 Gy leg-out partial body irradiation (leg-out PBI) without and with mitigators as marked. The data for 13 Gy leg-out PBI without ($n=10$) and with saline and Enrofloxacin are plotted over 160 days ($n=14$). Rats were given lisinopril ($\sim 24 \text{ mg m}^{-2} \text{ day}^{-1}$) started orally in the drinking water at 7 days after irradiation and continued to termination) along with subcutaneous injection of saline ($40 \text{ ml kg}^{-1} \text{ day}^{-1}$) from days 3-7 and Enrofloxacin ($10 \text{ mg kg}^{-1} \text{ day}^{-1}$) at days 2-28 ($n=13$ at start) and the same regimen with G-CSF administered subcutaneously at a dose of $100 \mu\text{g kg}^{-1}$ once a day starting 24 hours after radiation and continuing for 14 days ($n=13$ at start). Less than 20% of the groups given lisinopril with saline and Enrofloxacin with G-CSF ($p=0.01$) or without G-CSF ($p=0.002$) were morbid by 150 days as compared to 100% of rats given saline and Enrofloxacin alone.

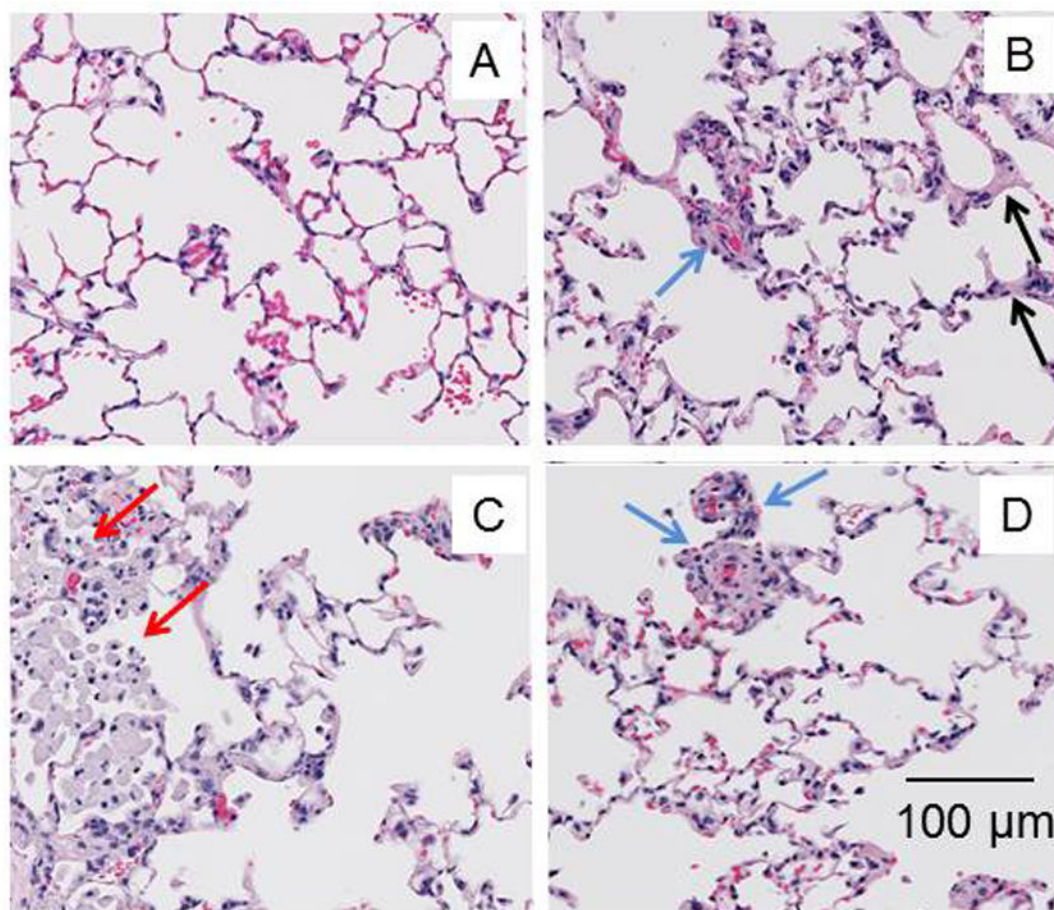


Fig. 6.

Histological evaluation of lungs in morbid rats during pneumonitis after 13 Gy leg-out PBI with partial shielding of one hind leg and given supportive care. Hematoxylin and eosin-stained lung sections from rats that were morbid in Fig. 5 between 42-70 days were scored as described in Methods. Typical images from matched non-irradiated lungs (from other studies) (A) and from irradiated lungs are shown in Figures B, C and D to point out thickened alveolar walls (B, see black arrows), thickened blood vessel walls (B and D, blue arrows) as well as macrophages (C, red arrows) are shown. Bar=100 μ m. Scores for all three histological end points were increased in morbid rats (see text for details).

Median and 20-80% values for blood urea nitrogen (BUN in mg dl⁻¹) at 90, 120 and 150 days after irradiation

Table 1

Groups given 13 Gy leg-out PBI plus	Number of rats	BUN (mg/dl) @ (Median with 20%-80% range)			<i>p</i> vs. 13 Gy leg-out PBI plus saline & Enrofloxacin
		90 days	120 days	150 days	
Saline & Enrofloxacin	10	64(49-70)	92(71-118)	120(120-120)	
Lisinopril & salineSt Enrofloxacin	11	28(25-29)	28(24-40)	41(39-48)	0.0001 for each time point
Lisinopril & salineSt Enrofloxacin & G-CSF	10	30(22-35)	30(26-37)	32(30-45)	0.0004 for each time point